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Age-Related Reductions in [^{3}H]WIN 35,428 Binding to the Dopamine Transporter in Nigrostriatal and Mesolimbic Brain Regions of the Fischer 344 Rat[1]

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ABSTRACT

In the present study, we used the potent cocaine analog [^{3}H]WIN 35,428 to map and quantify binding to the dopamine transporter (DAT) within the dorsal striatum, nucleus accumbens, substantia nigra, and ventral tegmental area in young (6-month-old), middle-aged (12-month-old), and aged (18- and 24-month-old) Fischer 344 rats. Quantitative autoradiographic analysis of indirect [^{3}H]WIN 35,428 saturation curves revealed two-site binding for all four brain regions in every age group. The percentage of binding to the high- or low-affinity sites did not differ with age or region and was approximately 50%. However, significant age-related decreases in the overall density (B_{max}) of [^{3}H]WIN 35,428-binding sites were observed in the striatum, nucleus accumbens, substantia nigra, and ventral tegmental area. The B_{max} within all brain regions declined by more than 15% every 6 months, with the B_{max} in the aged (24-month-old) group being approximately half that measured in the young adult (6-month-old) group. Competition experiments indicated that nomifensine also exhibited two-site binding to the DAT in Fischer 344 rats. No consistent age-related differences in binding affinities were noted with either [^{3}H]WIN 35,428 or nomifensine. Taken together, these results support the hypothesis that functional DATs within the nigrostriatal and mesolimbic systems are down-regulated with age, without changing their affinity for ligands.

Dopamine transporters (DATs) are integral neuronal membrane proteins that function to terminate dopaminergic neurotransmission by the rapid reuptake of synaptic dopamine (DA) into dopaminergic neurons. Because DAT is the main mechanism for clearing extracellular DA, it is the primary element that regulates the intensity and duration of dopaminergic neurotransmission (Giros et al., 1996). The uptake process is important for normal brain function because the administration of drugs that block the uptake process have dramatic behavioral and physiological effects (Ritz and Kuhar, 1993). Because it is responsible for the translocation of neurotoxins that can cause parkinsonian symptoms, DAT is also implicated in age-related neurodegenerative disorders, such as Parkinson's disease (Edwards, 1993).

Dramatic age-related deficits in the in vivo function of the DAT have been documented in aged animals. Significant reductions in the clearance rate of endogenous DA were measured using in vivo electrochemical methods in middle-aged and aged Fischer 344 (F344) rats and middle-aged nonhuman primates (Friedemann and Gerhardt, 1992; Gerhardt et al., 1995; Hebert and Gerhardt, 1998). Using the same technique, age-related differences in the capacity to clear extracellular DA were observed, with the maximum rate of DA transport being reduced by 50% in the striatum and nucleus accumbens of 24-month-old F344 rats (Hebert and Gerhardt, 1999). It has been speculated that changes in the density of functional DATs may be the basis for observed changes in capacity and rate of DA uptake (Hebert and Gerhardt, 1999). Alternatively, age-related changes in DA uptake may reflect altered affinity of DAT for DA.

In vitro localization of DATs in brain tissue has been routinely performed using radioligand binding and hybridization assays. Radiolabeling of uptake inhibitors, which bind to a recognition site associated with the DAT, and of antisense cDNA probes, which hybridize with DAT mRNA, has created the opportunity for quantitative autoradiographic assessment of the density and distribution of DATs (Himi et al., 1995). Radioligand binding to the human DAT has been reported to decrease linearly with age (Volkow et al., 1994).

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ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; F344, Fischer 344.

Similarly, reports indicate that the expression of human DAT mRNA declines significantly with age (Bannon and Whitty, 1997). Although DAT mRNA was also found to be significantly reduced in aged rats (Himi et al., 1995), there is a lack of consensus regarding age-related changes in radioligand binding to the DAT in laboratory rats. In two studies involving aged rats, significant reductions were seen in [^{3}H]mazindol binding density and [^{3}H]GBR 12783 binding within the striatum (Araki et al., 1997). In contrast, in another study, a similar number of striatal [^{3}H]GBR 12935-binding sites were reported in aged and young rats (Inglefield and Richfield, 1992).

Differential binding characteristics of the various radioligands used may account for the observed inconsistencies in DAT-binding densities in aged rats. [^{3}H]GBR compounds and [^{3}H]mazindol have been reported to vary in specificity for the DAT, leading to differences in reported transporter density in various brain regions (Madras et al., 1989; Izenwasser et al., 1994; Pristupa et al., 1994). Furthermore, [^{3}H]GBR 12935, the radioligand used in the study in which no age-related differences in binding density were observed, binds not only to the DAT but also to a piperazine acceptor site (Richfield, 1991). We have chosen to use a highly selective ligand for the DAT, [^{3}H]WIN 35,428 ([^{3}H]2-β-carbomethoxy-3β-(4-fluorophenyl)tropane), to investigate differences in binding densities within the dopaminergic cell bodies and terminal regions in F344 rat brain. [^{3}H]WIN 35,428 has excellent qualities for autoradiographic studies. Its DAT binding characteristics and pharmacokinetic properties have been very well characterized in vitro, ex vivo, and in vivo in rodents (Haaparanta et al., 1996), primates (Kaufman and Madras, 1993), and humans (Laakso et al., 1998). The lack of an ester bond makes it relatively resistant to metabolism. It has good selectivity for DAT over other monoamine transporters (Aloyo et al., 1995), and it shows relatively low nonspecific binding in the brain (Kaufman and Madras, 1993). In membranes and intact sections from rodent brain, [^{3}H]WIN 35,428 has been shown to bind with nanomolar affinity in a reversible, saturable, and stereoselective manner (Izenwasser et al., 1994). In the only aging study to use [^{3}H]WIN 35,428 to date, in vivo accumulation of this ligand in the striatum of aged monkeys was reduced by more than 50% compared with young adult monkeys (Kaufman and Madras, 1993).

The purpose of these experiments was to evaluate age-related changes in the binding characteristics of DAT proteins in both the terminal and cell body regions of the nigrostriatal and mesolimbic systems in young, middle-aged, and aged F344 rats. Our studies addressed the following questions: 1) Are there age-related changes in the number of DATs? 2) Are there differences in DAT-binding affinities between the age groups? 3) Are the differences region dependent? To approach these questions, two experiments were performed using in vitro radioligand binding with quantitative autoradiographic analysis. In experiment 1, indirect saturation curves for [^{3}H]WIN 35,428 binding were generated. In a second experiment, we studied the displacement of [^{3}H]WIN 35,428 by the DA uptake inhibitor nomifensine. We have previously reported significant age-related deficits in nomifensine-induced behavior and in vivo uptake inhibition (Hebert and Gerhardt, 1998, 1999) and wanted to investigate further whether age-related alterations in the affinity of DAT for this ligand were related to functional changes.

Experimental Procedures

Animals. Male F344 young adult (6 months old, $n = 9$), middle-aged (12 months old, $n = 9$), and aged (18 months old, $n = 9$; and 24 months old, $n = 9$) rats were used for [^{3}H]WIN 35,428 indirect saturation experiments. Male F344 young adult (6 months old, $n = 6$) and aged (24 months old, $n = 6$) rats were used in the nomifensine competition experiments. All rats were obtained from the National Institute on Aging (Harlan Sprague-Dawley, Inc., Indianapolis, IN). Protocols for animal use were approved by the Institutional Animal Care and Use Committee. Animals were housed according to approved guidelines, with food and water available ad libitum.

Tissue Preparation. Rats were anesthetized with urethane (1.5 g/kg i.p.) and sacrificed by decapitation. The brains were rapidly removed, frozen in powdered dry ice, and stored at −80°C. For sectioning, the brains were allowed to equilibrate to −20°C for 1 h, mounted on a chuck, serially sectioned with a cryostat in 10-μm coronal sections, and thaw-mounted onto Fisherbrand Superfrost slides (Fisher Scientific, Pittsburgh, PA). The sections were cut at the levels of the striatum/nucleus accumbens and substantia nigra/ventral tegmental areas based on the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986). The slide-mounted sections were stored at −80°C until assayed.

Binding Assays and Quantitative Autoradiography. Optimal binding conditions for [^{3}H]WIN 35,428 were established in preliminary studies. For both the indirect saturation analysis and the nomifensine competition experiments, slides were preincubated in 30 mM sodium phosphate buffer, pH 7.4, for 10 min at 4°C. The assays consisted of 30 mM sodium phosphate buffer containing 0.32 M sucrose (Reith and Coffey, 1993), 3.7 nM [^{3}H]WIN 35,428, and 8 to 10 concentrations of unlabeled WIN 35,428 (0.3 nM to 1 μM) or nomifensine (0.3 nM to 10 μM). In both experiments, total binding was defined in the absence of added unlabeled drugs, and nonspecific binding was defined in the presence of benztropine (30 μM). The slides were incubated for 90 min at 4°C. Unbound ligand was removed by washing the sections twice for 1 min in 4°C buffer that did not contain sucrose, followed by a quick rinse in 4°C water to remove buffer salts. Excess water was removed immediately from the slide under a stream of cool air. The slides were dried on a slide warmer (55°C) and then stored overnight at room temperature.

The next day, the slide-mounted brain sections were apposed to ^{3}H-labeled Hyperfilm (Amersham Corp., Arlington Heights, IL) along with calibrated ^{3}H-labeled microscales (Amersham Corp.) in film cassettes. Sections containing the striatum and nucleus accumbens were exposed to film for 12 days, and sections containing the substantia nigra and ventral tegmental area were exposed for 6 weeks. The films were developed for 4 min with Kodak D19 (Rochester, NY), rinsed for 45 s in an acid stop bath (Kodak Quick Stop), fixed for 4 min (Kodak Fixer), and rinsed for 10 min in distilled water.

Densitometry. Regional radioligand labeling intensities were quantified from the films using an MCID M4 Image Analysis System (Imaging Research, Inc., St. Catherine's, Ontario, Canada). A standard curve was generated for each piece of film by digitizing the autoradiograms of the standards. The nanocurie per milligram values used for calculations were provided with the ^{3}H standards (Amersham Corp.). Density measurements were made in quadruplicate by analyzing regions bilaterally in two brain sections from each animal. Specific binding (total − nonspecific) was more than 83% of total binding for all brain regions from all age groups.

Data Analysis. Indirect saturation curves were constructed using specific binding values for each ligand concentration. The curves were fit by nonlinear regression curve algorithms for one- and two-site binding using Prism software (GraphPAD Software, Inc., San Diego, CA). All curves were analyzed first as one-site binding and subsequently as two-site binding. The two-site binding fits were accepted only if the F test comparing the sum of squares for error was significantly reduced using the more complicated (two-site)

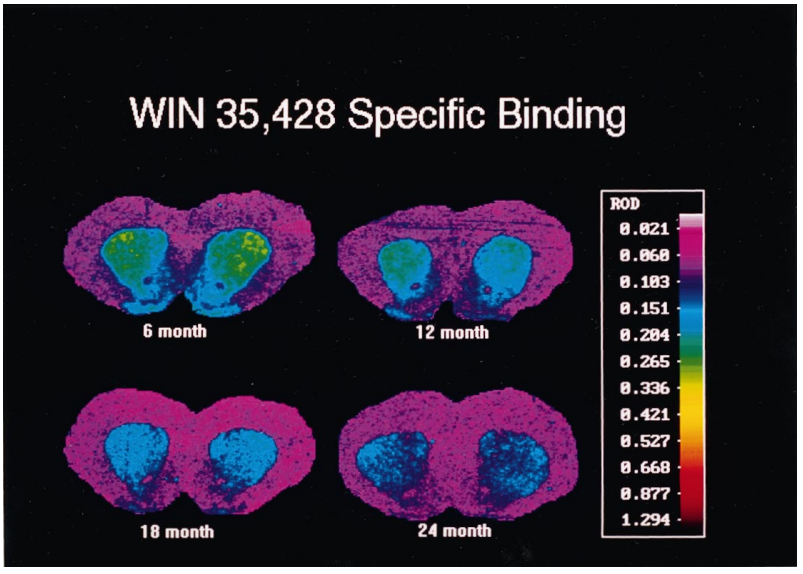

Fig. 1. Digitized pseudocolor images showing localization of specific [^{3}H]WIN 35,428 binding at the level of the dorsal striatum and nucleus accumbens in 6-, 12-, 18-, and 24-month-old F344 rats. In the computer-generated difference images of specific binding, an image of the nonspecific binding (30 μM benztropine) was subtracted from the image of total binding (3.7 nM [^{3}H]WIN 35,428). Linear scale at the right was generated from the standard curve. In these brain sections, it is apparent that binding is highest in the young adult (6 months) striatum/nucleus accumbens and decreases with age.

model. IC_{50} values and percent high-affinity sites were determined from the curve fits. B_{max} values were derived using the formula $B_{max} = [B_0 \cdot IC_{50}(high)/L + (B_0 \cdot IC_{50}(low)/L]$, where B_0 is the amount of specific binding obtained with L, IC_{50} is the concentration of unlabeled ligand that displaced half of the specific radioligand binding at either the high- or low-affinity site, and L is the concentration of radioligand (DeBlasi et al., 1989).

Statistical Analysis. Data are expressed as mean ± S.E.M. values (n = number of rats). Two-way analysis of variance (age × region) with Tukey-Kramer post-hoc analysis (GraphPAD Software, Inc.) was performed to identify statistical differences in the density and affinity of DAT by region and age. Significance level for the statistical tests was set at $p < .05$.

Materials. Nomifensine maleate, benztropine methane sulfonate, and WIN 35,428 were purchased from Research Biochemicals International (Natick, MA). [^{3}H]WIN 35,428 (83.5 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). All other reagents were of research grade.

Results

The specific binding of [^{3}H]WIN 35,428 was most prominent in nigrostriatal (substantia nigra and dorsal striatum) and mesolimbic (ventral tegmental area and nucleus accumbens) cell bodies and terminals in all animal age groups. Nonspecific binding, defined in the presence of 30 μM benztropine, was negligible (<18%; data not shown). WIN 35,428 (1 μM) displaced [^{3}H]WIN 35,428 binding to the same extent as 30 μM benztropine in all brain regions. Representative autoradiographic images showing the relative density and distribution of [^{3}H]WIN 35,428-binding sites in the striatum/nucleus accumbens of young (6-month-old), middle-aged (12-month-old), and aged (18- and 24-month-old) F344 rats are shown in Fig. 1. Within each age group, the density of [^{3}H]WIN 35,428 binding corresponded to the regional density of dopaminergic innervation, with the regional rank order of dorsal striatum > nucleus accumbens > ventral tegmental area ≥ substantia nigra.

The density and affinity of DAT-binding sites labeled by [^{3}H]WIN 35,428 were evaluated in saturation experiments using a fixed concentration of [^{3}H]WIN 35,428 (3.7 nM) and increasing concentrations of unlabeled WIN 35,428 (0.3 nM to 1 μM). Figure 2 depicts nonlinear regression curves generated from specific [^{3}H]WIN 35,428 binding data measured in the dorsal striatum of the four age groups of F344 rats. When the binding data were fit to either a one- or two-site model, the two-site model was statistically preferred for all regions in all age groups studied ($p < .01$, Table 1). Each site was found to represent approximately half of the total binding sites (51 ± 4%), regardless of brain region or age.

Inhibition constants (IC_{50}) for the high-affinity [^{3}H]WIN 35,428-binding sites ranged from 1.33 to 5.55 nM, and IC_{50} values for the low-affinity sites ranged from 164 to 404 nM. Age-related reductions (45%) in the affinity of the high-affinity binding site were noted in the striatum of rats aged 18 and 24 months [compared with 6-month-old rats, $F(3,32) = 4$, $p < .05$]. Significant, age-related increases (34–67%) in the affinity of the high-affinity binding site were observed in the substantia nigra of middle-aged and aged F344 rats [12, 18, and 24 months old versus the 6-month-old group, $F(3,30) = 5$,

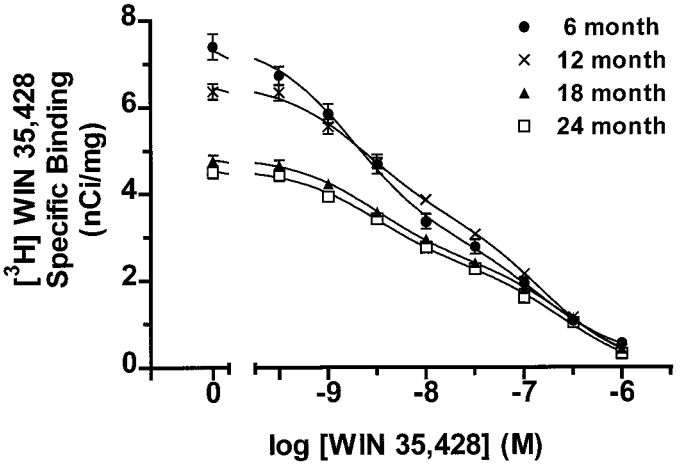

Fig. 2. Age-related decreases in the density of DAT-binding sites in dorsal striatum. Indirect saturation curves were generated with 3.7 nM [^{3}H]WIN 35,428. Specific binding is the difference in binding in the absence and presence of 30 μM benztropine. Data were analyzed using quantitative autoradiography and nonlinear curve fitting. Computer modeling of these curves supported a two-site binding model ($p < .01$). IC_{50} (high and low) and B_{max} values from this analysis are reported in Table 1. Data shown are the mean ± S.E.M. of nine rats per group.

TABLE 1
[3H]WIN 35,428 binding parameters from quantitative autoradiographic analysis

See Fig. 2 for details. In all brain regions of each age group studied, the two-site binding model was statistically preferred ($p < .01$). See *Experimental Procedures* for B_{max} calculation. Each value is mean ± S.E.M. from seven to nine rats per group.

Region (*n*)	IC_{50}		B_{max}	High-Affinity Site
	High	Low		
	nM		*pmol/mg*	%
Striatum				
6 mo (9)	1.79 ± 0.28	289 ± 59	2.78 ± 0.60	59 ± 1
12 mo (9)	2.37 ± 0.30	226 ± 30	2.27 ± 0.22	46 ± 1
18 mo (9)	3.27 ± 0.45*	164 ± 20	1.44 ± 0.09*	46 ± 1
24 mo (9)	3.29 ± 0.47*	190 ± 37	1.33 ± 0.21*	45 ± 2
Nucleus accumbens				
6 mo (9)	2.77 ± 0.28	221 ± 16	2.36 ± 0.17	43 ± 2
12 mo (9)	4.50 ± 0.40	215 ± 28	2.04 ± 0.28	48 ± 2
18 mo (9)	5.55 ± 1.40	240 ± 46	1.61 ± 0.28	47 ± 2
24 mo (9)	4.26 ± 0.72	185 ± 26	1.41 ± 0.17*	42 ± 2
Substantia nigra				
6 mo (7)	2.22 ± 0.32	301 ± 67	1.09 ± 0.20	49 ± 1
12 mo (9)	1.36 ± 0.13*	331 ± 55	0.64 ± 0.09*	59 ± 1
18 mo (9)	1.33 ± 0.17*	202 ± 21	0.40 ± 0.05***	59 ± 1
24 mo (7)	1.66 ± 0.07*	195 ± 19	0.29 ± 0.03***	62 ± 1
Ventral tegmental area				
6 mo (7)	1.71 ± 0.19	404 ± 64	1.25 ± 0.17	61 ± 1
12 mo (9)	1.68 ± 0.17	339 ± 59	0.86 ± 0.18	55 ± 2
18 mo (9)	1.90 ± 0.21	288 ± 43	0.47 ± 0.04**	59 ± 1
24 mo (7)	1.50 ± 0.15	250 ± 22	0.42 ± 0.07**	57 ± 1

Significance is denoted for within-region comparisons with 6-month-old rats: * $p < .05$, ** $p < .01$, *** $p < .001$.

$p < .05$]. IC_{50} values for the low-affinity component were consistent across brain regions and were unaltered by the aging process.

In contrast to the lack of age-related changes in affinity, we observed significant reductions in DAT density with age. There was a 10-fold range of overall B_{max} values derived from the curve fitting with the highest value in the striatum of 6-month-old rats and the lowest in the substantia nigra of 24-month-old rats (Table 1). In comparing the [3H]WIN 35,428 B_{max} values across the four age groups, a progressive age-related decline in the maximum number of DAT-binding sites was observed in all four brain regions [Table 1, $F(3,32) > 4$, $p < .05$]. The B_{max} values within all brain regions declined by more than 15% every 6 months, with the density of DATs in the aged (24-month-old) group being approximately half that measured in the young adult (6-month-old) group. In all regions except the nucleus accumbens, the deficits in B_{max} were significant at 18 and 24 months, and within the substantia nigra, significant reductions (41%) were found as early as 12 months and continued to decline with age. The age-related decreases, expressed as a percentage, showed that the densities of DATs on cell bodies (substantia nigra and ventral tegmental area) were more affected by age than those on the nerve terminals (striatum and nucleus accumbens). Likewise, age-related declines in DATs were greater in the nigrostriatal system (41–73% decline in the substantia nigra and 18–52% decline in the striatum) than in the mesolimbic system (31–66% decline in the ventral tegmental area and 13–40% decline in the nucleus accumbens).

Nomifensine (0.3 nM to 10 μM) also inhibited [3H]WIN 35,428 binding in a complex manner (Fig. 3). Results from this experiment confirmed age-related differences in the

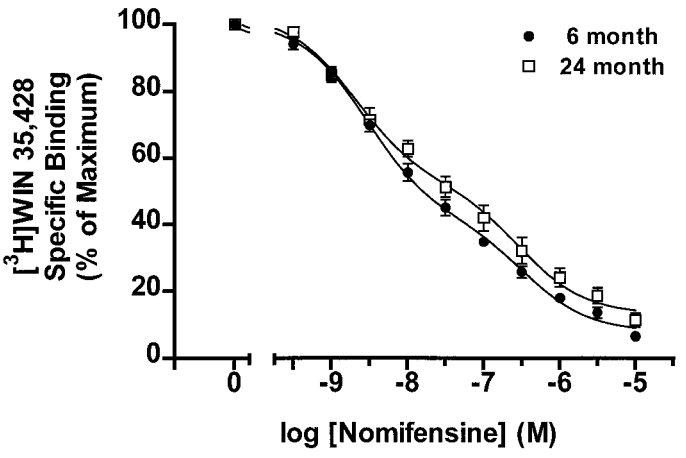

Fig. 3. Aging does not affect nomifensine inhibition of specific [3H]WIN 35,428 binding in the striatum (see Fig. 2 for details). [3H]WIN 35,428 binding is expressed as percent of maximal specific binding to normalize for differences between the two age groups (6 months, 6.2 ± 0.4 nCi/mg; 24 months, 4.8 ± 0.3 nCi/mg). Computer modeling of these curves supported a two-site binding model ($p < .01$). IC_{50} values from this analysis are reported in Table 2. Data shown are the mean ± S.E.M. of six rats per group.

maximal specific binding of [3H]WIN 35,428 in that the specific binding in the absence of nomifensine in the 6-month-old F344 rats was greater (35–50%) than that in the 24-month-old group for all brain regions examined (data not shown). Because of these age-related differences in maximal [3H]WIN 35,428 binding, data obtained from nomifensine competition studies were normalized as the percent of maximal binding in each region of 6- and 24-month-old rats before curve fitting. Nonlinear curve-fitting analysis showed that nomifensine displaced [3H]WIN 35,428 binding from two classes of binding sites, each of which again constituted approximately half of the specific binding sites (Fig. 3). IC_{50} values for the high-affinity nomifensine-binding sites ranged from 1.79 to 3.49 nM, whereas IC_{50} values for the low-affinity sites ranged from 179 to 508 nM (Table 2). No significant differences in either the high or low IC_{50} values were observed between the young (6-month-old) rats and the aged (24-month-old) F344 rats in any of the four brain regions studied.

TABLE 2
Nomifensine binding parameters from quantitative autoradiographic analysis

See Fig. 3 for details. In all brain regions of both age groups studied, the two-site binding model was statistically preferred ($p < .01$).

Region (*n*)	IC_{50}		High-Affinity Site
	High	Low	
	nM		%
Striatum			
6 mo (6)	3.49 ± 0.41	498 ± 63	61 ± 1
24 mo (6)	2.37 ± 0.32	403 ± 55	54 ± 1
Nucleus accumbens			
6 mo (6)	3.20 ± 0.33	508 ± 82	62 ± 1
24 mo (6)	2.74 ± 0.37	323 ± 34	64 ± 3
Substantia nigra			
6 mo (6)	3.23 ± 0.42	310 ± 56	52 ± 3
24 mo (6)	2.78 ± 0.15	434 ± 41	56 ± 1
Ventral tegmental area			
6 mo (6)	2.11 ± 0.35	274 ± 55	48 ± 2
24 mo (6)	1.79 ± 0.17	179 ± 36	47 ± 3

Each value is mean ± S.E.M. from six rats per group.

Discussion

The results of the present study indicate a progressive age-related decline in DAT-binding density within the F344 rat nigrostriatal and mesolimbic terminal and cell body regions. The decrease in the number of DAT binding sites in the striatum of aged rats (52% between 6 and 24 months) was slightly lower than that found in humans (Volkow et al., 1994) and more than that previously measured in mice (Sershen et al., 1985). However, the decrease observed here was equivalent to the decline observed in F344 rats using [^{3}H]mazindol (Shimizu and Prasad, 1991). The decreases in DAT binding that occur in aged F344 rats also correspond with the reported 40 to 75% deficits in mRNA expression found in rats (Himi et al., 1995) and humans (Bannon and Whitty, 1997). Similarly, the regional variation in DAT density observed in the present study confirms reports demonstrating higher DAT binding within the striatum compared with the nucleus accumbens (Hurd et al., 1994) and substantially lower density of DAT binding in the midbrain compared with striatal regions (Chen et al., 1996).

Using an array of different experimental paradigms, it has been well established that dopaminergic neuronal function deteriorates with senescence (Missale et al., 1986; Hebert and Gerhardt, 1998). In a previous investigation using four age groups of animals and in vivo electrochemistry, we documented progressive declines in both the capacity and rate of DA uptake in the striatum and nucleus accumbens of 24-month-old F344 rats compared with 6-month-old animals (Hebert and Gerhardt, 1999). The deficits in functional DA uptake within the striatum were noted as early as 12 months, and DAT binding levels were reduced by more than 50% by 24 months of age. In the same study, regional differences in the effects of age were observed, with the nigrostriatal neurons showing greater age-related declines in DA uptake than the mesolimbic neurons. In a study investigating age-related changes in [^{3}H]DA uptake, deficits of the same magnitude (~50%) were reported in DA uptake within the striatum of aged rats (Shimizu and Prasad, 1991), but because only two age groups of rats were used, the progressive nature of the decline could not be evaluated. Therefore, the reductions in DAT density we report in the present study coincide with the time course and regional variation of age-related deficits in DA uptake measured in vivo using electrochemical methods and in vitro using [^{3}H]DA uptake.

Changes in the density of DAT during aging may be a consequence of either a degeneration of DA neurons or a decrease in the relative number of DATs located on each neuron. Quantitative analysis of DA tissue levels in the nigrostriatal and mesolimbic systems of aged rats suggests that a decrease in DAT density is not the result of an age-related decrease in DA content (Hebert and Gerhardt, 1998). In addition, studies involving nonstereological cell-counting techniques of tyrosine hydroxylase-immunoreactive neurons have reported modest (~20–30%) age-related declines in striatal DA neurons of the rat (Fernandez-Ruiz et al., 1992), which may contribute in part to decreased DAT density. Clearly, these declines in DA neurons and little change in DA content do not parallel the ~50% reduction seen in DAT density in the present study. Regardless of the relationship of DATs to DA neurons in aged rats, reductions in the number of functional DATs would be expected to have significant functional consequences, particularly in the regulation of dopaminergic neurotransmission (Hornykiewicz, 1983). Prior studies from our laboratory have demonstrated significant age-related reductions in stimulus-evoked release and locomotor behavior that were concomitant with decreases in DA uptake (Hebert and Gerhardt, 1998, 1999). The diminution in DAT density with age may reflect a compensatory mechanism to maintain a certain level of dopaminergic neurotransmission despite the age-related reductions in DA release (Snyder et al., 1990). Likewise, down-regulation of the density of DAT in the aged brain may be a protective mechanism to limit further degeneration of dopaminergic neurons by neurotoxins that enter the neurons via the DAT (Romero-Ramos et al., 1997). The relationship between density of DATs and susceptibility to degeneration via translocation of neurotoxic agents remains unclear.

In this study, [^{3}H]WIN 35,428 bound to two sites regardless of the brain region and age group examined, demonstrating that two binding components for WIN 35,428 may represent an intrinsic property of the DAT that is not altered by age. Similarly, the relative distribution of binding between the two sites (~50%) remained constant between brain regions and age groups. This observation conflicts with other reports of [^{3}H]WIN 35,428 two-site binding, which have reported a relatively low percentage of binding at the high-affinity site in aging (Gracz and Madras, 1995). However, the lack of any consistent age-related changes in either the high or low IC_{50} values for [^{3}H]WIN 35,428 binding was not surprising because other reports have found no major age-related differences in DAT affinity (Shimizu and Prasad, 1991; Inglefield and Richfield, 1992; Volkow et al., 1994; Gracz and Madras, 1995; Himi et al., 1995; Araki et al., 1997). The lack of an effect of age on nomifensine binding affinity was interesting in that researchers in our laboratory have previously documented age-related declines in nomifensine-modulated locomotion (Hebert and Gerhardt, 1998), as well as age-related deficits in the ability of nomifensine to modulate DA clearance in vivo (Friedemann, 1992; Hebert and Gerhardt, 1999). Consequently, the results of the present study, combined with our prior in vivo investigations, support the conclusion that the age-related decline in the efficacy of nomifensine to inhibit DA uptake is not due to a decrease in its affinity for the DAT (Nakachi et al., 1995).

[^{3}H]WIN 35,428 binding to the DAT was first investigated in primate striatum, which was reported to contain both high- and low-affinity binding components (Madras et al., 1989). The experiments that followed using [^{3}H]WIN 35,428 binding produced mixed results. Several groups have reported two components for [^{3}H]WIN 35,428 binding to rat striatum (Richfield, 1991; Izenwasser et al., 1994), whereas others have observed only one component (Xu et al., 1995; Little et al., 1996). In reconciling these reported differences, two lines of evidence suggest that species and strain differences may be as, or even more, important for observing high- and low-affinity components for DAT binding. First, [^{3}H]WIN 35,428 binding experiments in mouse brain sections performed under the exact conditions as the present study resulted in only one component (Dickinson et al., 1999). Second, other studies in F344 rats have reported two DAT binding sites for [^{3}H]GBR 12935 (Inglefield and Richfield, 1992). This issue needs further investigation.

The explanation of two coexisting binding sites for the DAT

remains speculative. Currently, there is no evidence for a second DAT gene or for alternative splicing of DAT mRNA that may be responsible for the observed differences. A likely explanation may involve differences in post-translational modifications of the DAT (Gracz and Madras, 1995). The DAT is highly phosphorylated, and it has been shown that the phosphorylation state of the protein can affect its affinity for [3H]WIN 35,428 (Kitayama et al., 1994) and other ligands (Vrindavanam et al., 1996). Whether the nonidentity of DAT binding sites is truly a manifestation of some post-translational regulatory event (i.e., phosphorylation or accessory binding protein) or is caused by the existence of multiple molecular forms of the DAT is currently unknown.

Age-related changes in DAT density parallel the temporal changes in other dopaminergic system markers, including DA uptake itself. Nomifensine competition studies indicated that altered binding affinity cannot account for age-related changes in in vivo efficacy. Clarification of age-dependent alterations in the density of the DAT provides important information about the aging of dopaminergic systems and the regulation of age-related changes and provides clues regarding the potential age-related effects of drugs and toxins that act through the DAT. Furthermore, the progressive age-related decline in DAT density in the nigrostriatal and mesolimbic dopaminergic neurons, as demonstrated with [3H]WIN 35,428 binding, indicates the importance of age-matched subjects in behavioral and biochemical studies.

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